

# Mate Choice by Non-Virgin Females Contributes to Reproductive Isolation between Populations of the Harlequin Beetle-Riding Pseudoscorpion

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## Abstract

Determining whether reproductive isolation evolves through mate choice and/or gametic factors that prevent fertilization or through the post-zygotic mechanisms of hybrid sterility or inviability is fundamental to understanding speciation. Investigation of the pre- and post-zygotic components of reproductive isolation is facilitated in the pseudoscorpion, *Cordylochernes scorpioides*, by its indirect method of sperm transfer and viviparous embryonic development. Previous research on this species, in which mate discrimination was assessed in virgin females, suggested that female choice played only a minor role in reproductive isolation between populations from French Guiana and Panamá. Here, in a study of three allopatric populations of *C. scorpioides* from Panamá, we assessed mating-stage isolation in both virgin and once-mated females, and found that female discrimination depends critically on mating status. Virgin females were almost invariably receptive, showing no tendency to discriminate against males from allopatric populations. By contrast, non-virgin females were significantly more likely to reject foreign males than males from their own population. Male sexual motivation could not account for differences in either female sexual receptivity or male success in sperm transfer. Allopatric and sympatric males did not differ in number of spermatophores deposited as either a female's first or second mate. Nonetheless, allopatric males achieved significantly lower sperm transfer success not only with choosy, non-virgin females but also with virgin females. Given the lack of behavioral discrimination by virgin females, female receptivity was not the only factor influencing differences in sperm transfer success. Multivariate analysis of spermatophore morphology suggests that the higher failure rate of matings between allopatric males and virgin females resulted from population differences in sperm packet architecture. Overall, our findings indicate that assessment of discrimination against allopatric males that is limited to virgin females may seriously underestimate the contribution of female mate choice to reproductive isolation between populations.

## Introduction

Speciation occurs when populations diverge sufficiently to become reproductively isolated, but is this isolation initiated by pre-fertilization barriers to gene

flow or by post-zygotic genetic incompatibility? There is currently little consensus regarding the answer to this question, and much debate over the relative importance of genetic drift, natural selection, divergent sexual selection, sexual conflict and selfish

genetic elements in the speciation process (Mayr 1963; Lande 1981; Barraclough et al. 1998; Hurst & Schilthuizen 1998; Rice 1998; Turelli 1998; Hurst & Werren 2001; Coyne & Orr 2004; van Doorn et al. 2004; Arnquist & Lowe 2005; Rundle & Nosil 2005; Telschow et al. 2005; Parker 2006; Hayashi et al. 2007). Determining how reproductive isolation takes place, i.e. whether through mate choice and/or gametic barriers that act to prevent fertilization or through the post-zygotic mechanisms of hybrid sterility or inviability, is fundamental to understanding the speciation process (Markow 1997; Coyne & Orr 1998).

As Coyne & Orr (1989: 363) pointed out in their classic study of speciation in *Drosophila*: 'We would like to know which type of isolation (pre- or post-zygotic) is most important in reducing gene flow between incipient species, for this factor would be the primary component of speciation'. Nonetheless, a unifying theory of speciation has remained elusive, perhaps because patterns in nature exhibit profound differences between lineages in the relative rates at which pre- and post-zygotic isolation evolve. Data on the relationship between divergence time and hybridizability across vertebrate clades (Wilson et al. 1974; Zeh & Zeh 2000) have shown that bird and frog species pairs retain the capacity to produce viable hybrid offspring for up to 60 Myr, an order of magnitude longer than mammals. Embryo transfer experiments and observations of spontaneous abortion and female death during pregnancy have demonstrated that the inability of mammalian species to produce hybrids frequently results from fetomaternal incompatibility rather than from incompatibility at the pre-fertilization stage (Gray 1972; Kraemer 1983; Tate et al. 1997; Vrana et al. 1998; Vrana 2007).

To account for the observed differences in the hybridizability of vertebrate lineages, Zeh & Zeh (2000) proposed that the reproductive stage at which divergence occurs most rapidly between populations is strongly influenced by whether a species is oviparous or viviparous. In viviparous species, development of the embryo within the mother creates a post-fertilization arena for forms of genomic conflict absent in species that lay eggs (Haig & Westoby 1989; Moore & Haig 1991; Zeh & Zeh 2001). Viviparity-driven conflict should generate perpetual antagonistic coevolution (Rice & Holland 1997) between: (1) individual embryos within the mother, (2) the mother's reproductive physiology and the developing fetus, and (3) paternally and maternally inherited genomes within individual embryos (Haig

1993; Spencer et al. 1999; Crespi & Semeniuk 2004). Interactions between embryos and the maternal uterine environment are therefore predicted to diverge rapidly between populations and to frequently represent the primary post-zygotic barrier to hybridization (Zeh & Zeh 2000). In addition, by generating genetic incompatibility within populations, viviparity-driven conflict is likely to favor the evolution of polyandry and reduced reliance on mate choice, thereby limiting the potential for divergence at the pre-copulatory stage (Zeh & Zeh 2001). This combination of rapid coevolution between embryos and mothers and restrained pre-copulatory divergence should amplify the importance of post-zygotic isolation as a speciation mechanism among viviparous taxa.

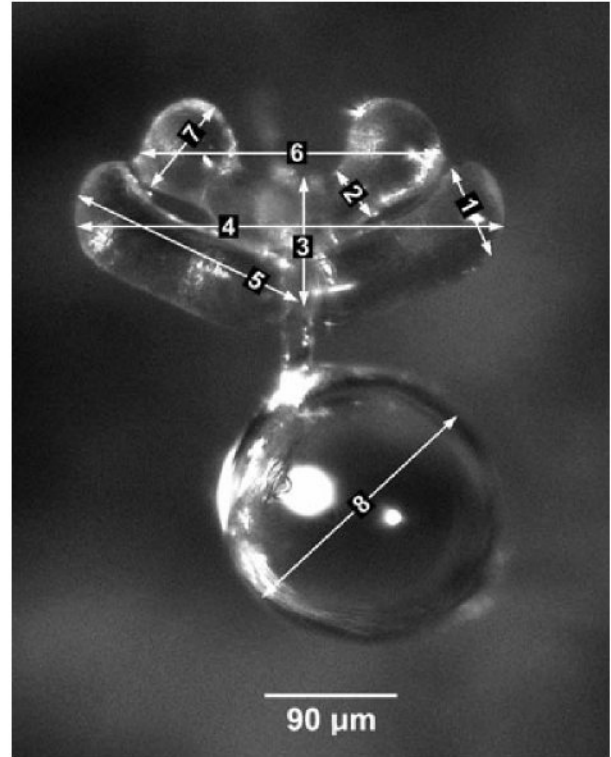
Although studies to quantify relative rates of divergence across the mating, gametic, and post-zygotic stages of reproduction have been carried out on a range of egg-laying taxa (Coyne & Orr 1989, 1997; Hewitt et al. 1989; Wade et al. 1994; Sasa et al. 1998; Howard 1999; Price 1997), there have been few comprehensive investigations of reproductive isolation in viviparous species. Here, as part of an ongoing investigation of speciation mechanisms in a live-bearing arthropod, we present the results of a study of mating-stage components of reproductive isolation between three allopatric populations of the harlequin beetle-riding pseudoscorpion, *Cordylocheres scorpioides*, from the Republic of Panamá.

Originally classified as four species based on male sexually dimorphic traits, *C. scorpioides* was subsequently reclassified as a single species, ranging from Costa Rica to northern Argentina (Beier 1948). However, mtDNA COI sequence data (Wilcox et al. 1997; Zeh et al. 2003), multilocus DNA profiling (Zeh et al. 1992), protein electrophoresis (Zeh & Zeh 1994), and reproductive compatibility experiments (Zeh & Zeh 1994; J.A. Zeh, unpubl. data), indicate that *C. scorpioides* is actually a complex of cryptic species in which genetic divergence and post-zygotic incompatibility appear to have outpaced the evolution of pre-zygotic reproductive isolation. For example, crosses between populations from central Panamá and French Guiana produced hybrid embryos that were invariably inviable (Zeh & Zeh 1994). Despite this complete reproductive incompatibility at the post-zygotic stage, morphological divergence was restricted to traits of the spermatophore, and there was apparently little isolation between populations at the behavioral level. Whereas all hybrid embryos spontaneously aborted in the early stages of development, nearly 80% of virgin females accepted foreign

males as mating partners (Zeh & Zeh 1994). In the study reported here, we assess population divergence in spermatophore morphology and mating-stage isolation in both virgin and once-mated females. Our findings show that experiments limited to virgin females may significantly underestimate the importance of female mate choice as a reproductive isolating mechanism.

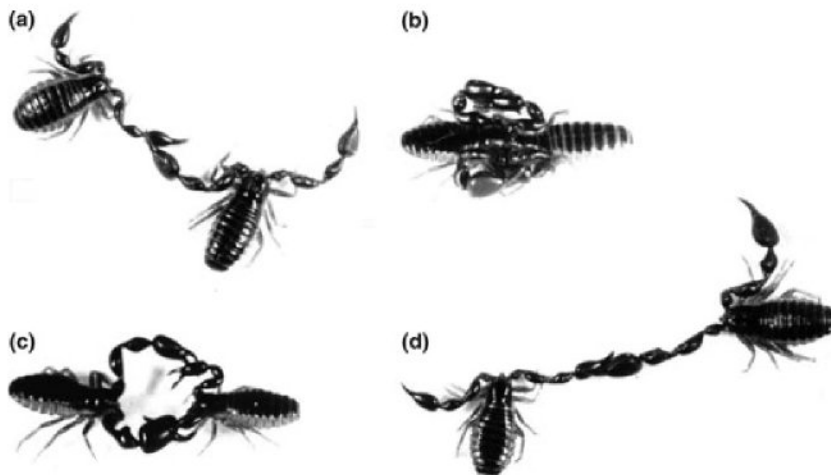
### Reproductive Biology of *Cordylochernes scorpioides*

As a viviparous arthropod, *C. scorpioides* possesses a suite of reproductive and behavioral traits that makes it highly amenable to partitioning reproduction isolation into pre- and post-zygotic components (Zeh & Zeh 1994). Embryos develop in a translucent brood sac overlying the female's genital aperture (Weygoldt 1969), and development can be easily monitored with minimal disturbance to mother or embryos. This 'external-womb' form of viviparity circumvents the technical difficulties of diagnosing early-stage spontaneous abortion in other viviparous species such as mammals. Mating behavior in the harlequin beetle-riding pseudoscorpion involves a well-defined sequence of behaviors (Fig. 1) in which the male grasps the female while he constructs and deposits a spermatophore on the substrate. The spermatophore consists of a stalk, a ball of fluid, and, at the apex of the stalk, a complex, folded, tubular packet containing the sperm (Fig. 2). After spermatophore deposition, the male maneuvers the female into a position in which the sperm packet directly contacts her gonopore. High-magnification video analysis (50X) has revealed that successful attachment of the sperm packet to the gonopore only occurs when complete spermatophores are deposited



**Fig. 2:** Top view of a spermatophore showing the measured traits of the sperm packet (1–7) and the ball of fluid (8). Trait abbreviations are as follows: (1) front wing maximum diameter (FWMAXD); (2) rear wing minimum diameter (RWMIND); (3) center length (CL); (4) front wing total length (FWTL); (5) front wing length (FWL); (6) rear wing total length (RWTL); (7) rear wing bulb diameter (RWBD), and (8) ball diameter (BD).

(stalk + ball of fluid + sperm packet) and is associated with a pronounced abdominal flexure by the female (Zeh & Zeh 1994). This flexure presses the sperm packet down onto the ball of fluid, causing a



**Fig. 1:** Mating sequence in which: (a) the male grasps the female's pedipalp to initiate mating; (b) holds her in a stationary position while depositing a spermatophore on the substrate; (c) reverses and attempts to pull the female over the spermatophore, and (d) maintains a grasp on the female during the sperm uptake phase. The male is on the right in (a) and (d) and on the left in (b) and (c).

long, hooked tube to evert from the packet into the female's genital aperture. The packet then detaches from the stalk and remains in position over the female's gonopore while sperm are evacuated into her reproductive tract. The emptied sperm packet takes on a characteristic flattened shape and is subsequently removed by the female rubbing her genital region against the substrate. In a typical mating event, the male produces his first spermatophore within 6 min of encountering a female. A lapse of approx. 15 min is then required between successive spermatophore depositions, during which the male attempts to maintain his hold on the female.

This indirect method of sperm transfer makes female cooperation essential for successful insemination (Zeh et al. 1998). Females typically accept only the first of two or more sperm packets deposited by a male during a single mating event (Zeh et al. 1998). Unreceptive females engage in one of several behaviors to block sperm transfer. In some cases, females aggressively resist the male and terminate mating by breaking free from the male's grasp before he initiates construction of a spermatophore. Alternatively, females occasionally refuse to remain stationary during spermatophore deposition, forcing the male to move and lose contact with the still incomplete spermatophore. Finally, in the majority of cases, the female cooperates with the male throughout the entire period of spermatophore construction and deposition, only to resist being pulled forward over the sperm packet (Zeh et al. 1998).

## Methods

Pseudoscorpions were collected from three locations in the Republic of Panamá: (1) Parque Nacional Soberanía in the former Canal Zone (CZ) of central Panamá (09°05'N/79°37'W), (2) western Chiriquí province (CHIR) near the border with Costa Rica (08°51'N/82°44'W), and (3) Bocas del Toro province (BDT) on the Caribbean (Atlantic) coast of western Panamá (09°00'N/82°16'W). The Isthmus of Panamá is transected by a central cordillera that reaches an elevation of 3000 m in western Panamá and decreases to 200 m in central Panamá. Our collections were made over a region spanning approx. 350 km from central (CZ) to western Panamá where sites were located on either the Atlantic (BDT) or the Pacific (CHIR) versant of the cordillera. Pseudoscorpions were collected from decaying trees (*Ficus* spp.) or were removed from harlequin beetles captured on newly dead or dying trees. Males and females for this study were either the laboratory-

reared progeny of field-inseminated females or field-collected nymphs that molted to sexual maturity in the laboratory. To ensure virginity, nymphs were reared individually, and provisioned with *Drosophila melanogaster* and *Tribolium confusum* larvae, as described elsewhere (Zeh et al. 2005).

## Mating-Stage Compatibility Experiment

Virgin females (n = 316) from the CZ (n = 125), BDT (n = 103) and CHIR (n = 88) populations were given the opportunity to mate with a randomly selected male (male A) from either their own population or one of the two allopatric populations. Within populations, no matings were carried out between siblings. Each replication was initiated by placing a female with a male in a 28-mm diameter mating arena. Behavioral interactions were videotaped (JVC GR-SXM330u super VHS camcorder, JVC Company of America, Wayne, NJ, USA) for 40 min under red fiber optics illumination. Immediately following the mating, both the female and the mating arena were examined at 20–60× magnification for the presence of emptied sperm packets and/or intact, i.e. unused spermatophores. Following this first mating opportunity, males and females were returned to their individual vials. The videotape of each mating was transcribed to determine the number of spermatophores deposited and the number of sperm packets successfully transferred by the male to the female. After approx. 48 h, each female that had accepted a sperm packet from a sympatric male A was then paired with an allopatric male B and vice versa. The second mating opportunity was set up, videotaped and transcribed, as described above.

The combination of external spermatophore deposition and diagnostic female behavior in *C. scorpioides* provides a unique, non-invasive window for assessing male sexual motivation, female sexual receptivity and male success in sperm packet transfer. Male sexual motivation toward sympatric and allopatric females was quantified on the basis of the number of spermatophores deposited by a male. A female was scored as sexually receptive, if she cooperated by allowing a male to pull her over at least one of his deposited spermatophores. In matings in which females were sexually receptive, males were scored as having successfully transferred sperm, if complete spermatophore deposition was followed by pronounced abdominal flexure by the female and an emptied sperm packet was subsequently found either attached to the female's gonopore or in the mating arena. A male could thus fail in sperm transfer either

because the female was sexually unreceptive or because his sperm packet did not successfully attach to the female's gonopore. Statistical analyses were carried out using SAS, v8.02 (SAS 2001). For analyses of the number of spermatophores deposited by males and the number of sperm packets accepted by females, Kruskal–Wallis non-parametric analyses of variance (ANOVAS) were carried out. Logistic regression and Fisher's exact tests were used to assess female and male population effects on female sexual receptivity and on success/failure of sperm transfer.

### Population Divergence in Spermatophore Morphology

To obtain spermatophores for morphometric analysis, males from each population were each placed in a 50 mm-diameter Petri dish with a virgin, sympatric-population female. Matings were observed at 12× magnification under a stereomicroscope. Immediately after spermatophore deposition, each male was prevented from pulling the female over the sperm packet. The pseudoscorpions were removed from the Petri plate which was immediately transferred to the stage of an Olympus SZ60 stereomicroscope (Olympus America Inc., Center Valley, PA, USA) for photomicroscopy of the top view of the spermatophore (126× magnification). A digital image of each spermatophore was recorded using an Olympus DPI2 digital camera. Only perfectly intact spermatophores, correctly oriented for accurate measurement, were included in the analysis ( $n = 17$  BDT, 21 CZ and 13 CHIR). Eight linear dimensions of each spermatophore were measured from these digital images, using the NIH program, Image, v1.62. Numbered traits (see Fig. 2) and their abbreviations are as follows: (1) front wing maximum diameter (FWMAXD); (2) rear wing minimum diameter (RWMIND); (3) center length (CL); (4) front wing total length (FWTL); (5) front wing length (FWL); (6) rear wing total length (RWTL); (7) rear wing bulb diameter (RWBD), and (8) ball diameter (BD). Population divergence in each spermatophore trait was quantified using univariate ANOVAS. To assess overall divergence in spermatophore morphology, we carried out both a multivariate analysis of variance (MANOVA) and a canonical discriminant analysis of the eight traits (SAS procedure, CANDISC).

### Results

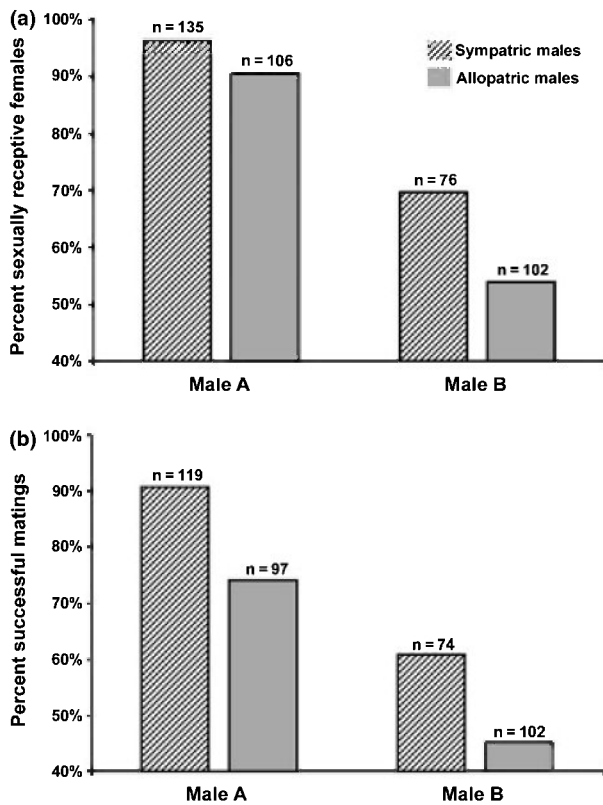
The percentage of females that were receptive to male As (Table 1) from their own population (CZ × CZ, BDT × BDT and CHIR × CHIR) was uniformly high ( $\geq 94\%$ ) and did not differ significantly

**Table 1:** Percentage of females from Canal Zone (CZ), Bocas del Toro province (BDT), and western Chiriqui province (CHIR) sexually receptive at first mating to males (male As) from each population and percentage of matings with successful sperm transfer

Female population	Male population	Percentage receptive	n	Percentage successful	n
CZ	CZ	96	54	90	48
	BDT	95	22	77	22
	CHIR	95	19	79	14
BDT	BDT	98	46	90	42
	CHIR	80	10	78	9
	CZ	96	23	77	22
CHIR	CHIR	94	35	93	29
	CZ	79	24	65	23
	BDT	100	8	63	8

between the three populations ( $3 \times 2$  Fisher's exact test;  $p = 0.7353$ ). Similarly, the percentage of successful first matings (Table 1) to sympatric males was high ( $\geq 90\%$ ) and was again independent of female source population ( $3 \times 2$  Fisher's exact test,  $p = 0.9245$ ). In first matings involving allopatric males (CZ × BDT, CZ × CHIR, BDT × CHIR, BDT × CZ, CHIR × CZ and CHIR × BDT), neither the percentage of females receptive nor the percentage of successful matings varied significantly between crosses ( $6 \times 2$  Fisher's exact test;  $p = 0.2467$  and  $p = 0.8722$ , respectively). Consequently, data from the nine population cross types were pooled into sympatric and allopatric male A categories for subsequent analyses. Analysis of the pooled data revealed no effect of male type (sympatric or allopatric) on female receptivity (Fig. 3a;  $2 \times 2$  Fisher's exact test;  $p = 0.1046$ ) but a significantly higher percentage of matings with successful sperm transfer for sympatric Male As (Fig. 3b;  $2 \times 2$  Fisher's exact test;  $p = 0.0010$ ). Although sympatric and allopatric males did not differ in the number of spermatophores deposited (Fig. 4a; Kruskal–Wallis non-parametric analysis of variance,  $\chi^2_1$  approximation = 0.0736;  $p = 0.7861$ ), the mean number of successfully transferred sperm packets was significantly higher for sympatric males (Fig. 4b;  $\chi^2_1$  approximation = 6.71;  $p = 0.0096$ ).

For females' second mating (Table 2), data were again pooled into allopatric and sympatric male categories based on the absence of heterogeneity within each male B category ( $3 \times 2$  and  $6 \times 2$  Fisher's exact tests;  $p \geq 0.6322$ ). For these non-virgin females, analysis of the pooled data revealed a significant advantage for sympatric males in terms of both female receptivity (Fig. 3a;  $2 \times 2$  Fisher's exact test;  $p = 0.0435$ ) and the percentage of matings with

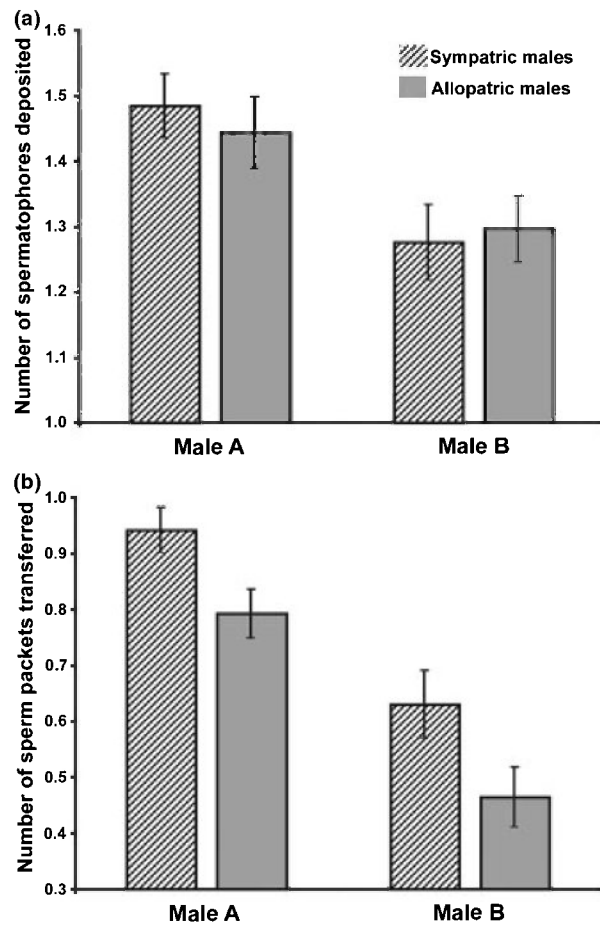


**Fig. 3:** Comparison of sympatric and allopatric males as females' first (male A) and second (male B) mates in terms of (a) the percentage of females that were sexually receptive and (b) the percentage of matings in which the male successfully transferred a sperm packet.

successful sperm transfer (Fig. 3b;  $2 \times 2$  Fisher's exact test;  $p = 0.0474$ ). As was the case for male As, sympatric and allopatric Male Bs did not differ in the number of spermatophores deposited (Fig. 4a;  $\chi^2_1$  approximation = 0.0467;  $p = 0.8290$ ), but the mean number of successfully transferred sperm packets was significantly higher for sympatric males (Fig. 4b;  $\chi^2_1$  approximation = 4.33;  $p = 0.0374$ ).

The validity of pooling data within the sympatric and allopatric mating categories was corroborated, using logistic regression. In all cases, the logistic regression and Fisher's exact tests were in agreement. That is, within the sympatric mating category, there was no effect of female population on either female receptivity or per cent successful sperm transfer ( $p \geq 0.6401$ ), and, within the allopatric mating category, there was no effect of female, male, or female  $\times$  male population on either female receptivity or per cent successful sperm transfer ( $p \geq 0.1878$ ).

These results indicate that, although allopatric males were less successful at transferring sperm



**Fig. 4:** Comparison of sympatric and allopatric males as females' first (male A) and second (male B) mates in terms of (a) the mean number of spermatophores deposited by a male and (b) the mean number of sperm packets accepted by the female. Plots are  $\pm$  SE.

**Table 2:** Percentage of females from Canal Zone (CZ), Bocas del Toro province (BDT), and western Chiriqui province (CHIR) sexually receptive at second mating to males (male Bs) from each population and percentage of matings with successful sperm transfer

Female population	Male population	Percentage receptive	n	Percentage successful	n
CZ	CZ	68	28	56	27
	BDT	52	27	44	27
	CHIR	57	14	36	14
BDT	BDT	76	25	68	25
	CHIR	67	9	44	9
CHIR	CZ	54	26	50	26
	CHIR	65	23	59	22
	BDT	47	17	47	17
	BDT	56	9	44	9

packets than sympatric males, both as male As and as male Bs, discrimination against allopatric males was only manifested in non-virgin females. To

**Table 3:** Results of univariate anovas of spermatophore traits

Trait	$r^2$	$F$	$p$	$p$ Bonf.
FWMAXD	0.236	7.10	0.002	0.006
RWMIND	0.192	5.45	0.007	0.007
CL	0.097	2.48	0.095	0.008
FWTL	0.094	2.39	0.103	0.010
FWL	0.086	2.16	0.127	0.012
RWTL	0.052	1.26	0.294	0.017
RWBD	0.049	1.19	0.313	0.025
BD	0.031	0.75	0.480	0.050

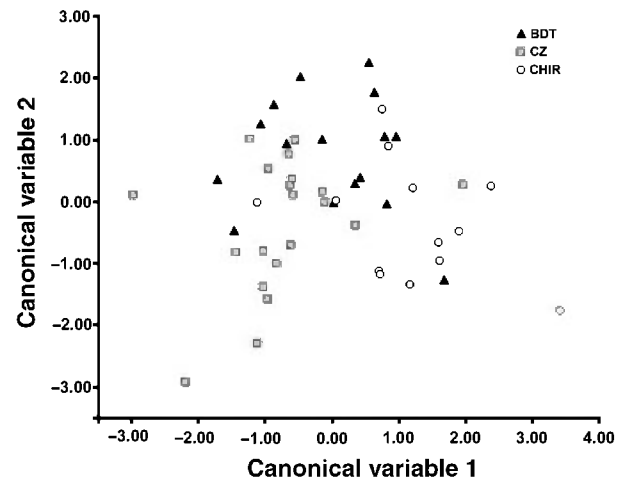
$r^2$ , proportion of trait variance attributable to population. Traits are ranked from most to least significantly different between populations; 'p Bonf.', critical p-values calculated using the sequential Bonferroni correction for the number of tests (see Rice 1998); FWMAXD, front wing maximum diameter; RWMIND, rear wing minimum diameter; CL, center length; FWTL, front wing total length; FWL, front wing length; RWTL, rear wing total length; RWBD, rear wing bulb diameter; (BD) ball diameter.

examine whether such discrimination may have involved the assessment of an allopatric male's ability to successfully transfer a sperm packet, we compared the percentage of successful matings for sympatric and allopatric males in a subset of the data that included only those cases in which females were sexually receptive. Not surprisingly, given the absence of discrimination by virgin females, the percentage of successful matings remained significantly lower for allopatric male As than for sympatric male As ( $2 \times 2$  Fisher's exact test;  $p = 0.0090$ ). By contrast, allopatric and sympatric male Bs did not differ significantly in their rates of successful sperm packet transfer to sexually receptive females ( $2 \times 2$  Fisher's exact test;  $p = 0.3986$ ), suggesting that discriminating, non-virgin females are able to assess the likelihood that an allopatric male can successfully transfer sperm.

The three populations exhibited significant divergence in two of the eight traits of the spermatophore (FWMAXD and RWMIND) when significance levels were adjusted for multiple comparisons (Table 3). For these two traits, approx. 20% of the total phenotypic variance was attributable to population. Despite some overlap in their discriminant function scores (Fig. 5), the populations were highly divergent in their multivariate mean values (Wilks' Lambda = 0.467;  $df = 16, 78$ ;  $p = 0.0093$ ).

## Discussion

The results of this study indicate that whether or not *C. scoriooides* females discriminate against males from foreign populations depends critically on female mat-



**Fig. 5:** Divergence between allopatric populations in spermatophore morphology as measured by canonical discriminant analysis. Abbreviations for populations are as follows: CZ, Canal Zone; CHIR, western Chiriqui province; BDT, Bocas del Toro province.

ing status. Virgin females were almost invariably sexually receptive in both homo- and hetero-population crosses, showing no significant tendency to discriminate against males from allopatric populations. By contrast, non-virgin females were significantly more likely to reject foreign males than males from their own population. Because unreceptive *C. scoriooides* females generally do not reveal their unwillingness to accept sperm until after the male has deposited a spermatophore, it was possible to reject male sexual motivation as an explanation for differences between allopatric and sympatric males in both female sexual receptivity and sperm transfer success. The two categories of males did not differ significantly in the number of spermatophores deposited either as the first or the second male to mate with a female. Nonetheless, allopatric males achieved a significantly lower rate of successful sperm transfer to both virgin and non-virgin females than did sympatric males. Given the lack of behavioral discrimination by virgin females, these findings demonstrate that female receptivity was not the only factor influencing male differences in sperm transfer success. Our multivariate analysis of spermatophore morphology suggests that the higher failure rate of matings between allopatric males and virgin females resulted, at least in part, from significant population differences in the architecture of the sperm packet. Such differences in sperm packet morphology are likely to hinder the correct positioning of the packet over the female's gonopore, impeding entry of the hooked tube into her reproductive tract.

The complex sperm packets of *Cordylochernes pseudoscorpions* are analogous to the male genitalia of organisms that transfer sperm directly (Eberhard 1985), and the findings reported here are consistent with a large body of evidence documenting rapid evolutionary divergence in male genital morphology (Eberhard 2004; Hosken & Stockley 2004).

Interestingly, divergence in spermatophore morphology appears not to have played a role in the lower sperm transfer success of allopatric males to non-virgin females. In the subset of the non-virgin female data that included only sexually receptive females, male population had no effect on the percentage of matings with successful sperm transfer. With non-virgin females, the lower sperm transfer success of allopatric males was entirely attributable to their higher rate of rejection by females. This result suggests that non-virgin females may use subtle behavioral, tactile or chemosensory cues to discriminate against allopatric males that are likely to be unsuccessful at transferring sperm.

It could be argued that apparent female discrimination against allopatric males at second mating resulted not from active female choice but from population divergence in seminal products that depress female sexual receptivity (see Rice & Holland 1997; Chapman et al. 2003; Liu & Kubli 2003; Sakaluk et al. 2006). According to this hypothesis, seminal products of allopatric males are less effective at inhibiting remating, and females mated first to an allopatric male are therefore more likely to remate, irrespective of male population at second mating. This effect could explain the observed pattern of higher female receptivity toward sympatric male Bs. However, this seems unlikely as the level of female receptivity toward sympatric male Bs reported here (70%) is almost identical to that observed in an intrapopulation study (69%), in which both male As and male Bs were sympatric (Zeh et al. 1998).

In this pseudoscorpion (Zeh et al. 1998), as in many other animal species, females encounter potential mates in a sequential fashion and do not have the opportunity to simultaneously evaluate a range of male phenotypes (Janetos 1980; Gabor & Halliday 1997; Schäfer & Uhl 2004). Under these conditions, a female must assess the current male against either an internal standard or her memory of males previously encountered (Janetos 1980). Sequential mate choice is thus an iterative decision-making process in which a female must repeatedly choose whether to mate or not. As Gabor & Halliday (1997) have pointed out, the sequential model of female choice is likely to be particularly important

in species, such as *C. scorpioides* (Zeh et al. 1997), in which females are polyandrous and can store sperm. For such species, Halliday (1983) hypothesized that, to ensure fertilization, virgin females should mate with the first male encountered. Once mated, females should then become discriminating. Although relatively few studies have explicitly investigated this hypothesis, empirical evidence from a wide range of taxa, including spiders (Watson 1991; Schäfer & Uhl 2004), newts (Gabor & Halliday 1997), and guppies (Pitcher et al. 2003), indicates that females do become more selective after first mating (but see Kodric-Brown & Nicoletto 2001). A recent mathematical model has shown that even a slight risk of never mating should favor a significant reduction in the level of choosiness among virgin females (Kokko & Maples 2005). These empirical and theoretical findings thus suggest that assessment of female preference based on virgin females may frequently underestimate the strength of female choice in natural populations.

The significant effect of mating status on female discrimination against allopatric males detected here in the harlequin beetle-riding pseudoscorpion may have two important general implications for empirical studies of speciation. First, comparison of the results of this study with previous investigations of *C. scorpioides* (Zeh et al. 1998) indicates that patterns of mate choice at the within-population level cannot be extrapolated to assess the potential effects of mating status on female discrimination against males from foreign populations. Within the CZ population of *C. scorpioides*, we have been unable to demonstrate mate choice by non-virgin females based on any aspect of male phenotype (Zeh et al. 1998). The only identifiable criterion used by females to accept or reject a mate is prior acceptance of a sperm packet from that male, with females actively discriminating against previous mates. Second, many investigations of behavioral isolation between allopatric populations or closely related species (Coyne & Orr 2004), including our own earlier study of *C. scorpioides* (Zeh & Zeh 1994), have been carried out using virgin females. If females forego choice at first mating in favor of ensuring a supply of sperm, assessment of mate discrimination limited to virgin females may seriously underestimate the relative contribution of female mate choice to overall reproductive isolation.

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